

# The compactness of ribonuclease A and reduced ribonuclease A

Jun-Mei Zhou<sup>a,\*</sup>, Ying-Xin Fan<sup>a</sup>, Hiroshi Kihara<sup>b</sup>, Kazumoto Kimura<sup>c</sup>, Yoshiyuki Amemiya<sup>d</sup>

<sup>a</sup>National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, PR China

<sup>b</sup>Physics Laboratory, Kansai Medical University, Uyamahigashi, Hirakata, Osaka 573, Japan

<sup>c</sup>Division of Medical Electronics, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan

<sup>d</sup>Faculty of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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**Abstract** The compactness of ribonuclease A with intact disulfide bonds and reduced ribonuclease A was investigated by synchrotron small-angle X-ray scattering. The  $R_g$  values and the Kratky plots showed that non-reduced ribonuclease A maintain a compact shape with a  $R_g$  value of about 17.3 Å in 8 M urea. The reduced ribonuclease A is more expanded, its  $R_g$  value is about 20 Å in 50 mM Tris-HCl buffer at pH 8.1 containing 20 mM DTT. Further expansions of reduced ribonuclease A were observed in the presence of high concentrations of denaturants, indicating that reduced ribonuclease A is more expanded and is in neither a random coil [A. Noppert et al., FEBS Lett. 380 (1996) 179–182] nor a compact denatured state [T.R. Sosnick and J. Trewhella, Biochemistry 31 (1992) 8329–8335]. The four disulfide bonds keep ribonuclease A in a compact state in the presence of high concentrations of urea.

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**Key words:** Ribonuclease A; Disulfide bond; Globularity; Small-angle X-ray scattering; Protein folding

## 1. Introduction

Ribonuclease A (RNase A) is one of the most extensively studied enzymes, with its sequence and three-dimensional structure being known completely. The course of its unfolding during denaturation by acid [1], heat [2], pressure [3] and denaturants [4–6], as well as its renaturation during refolding have also been well characterized in the literature [7–10]. In those refolding studies, RNase A denatured in 8 M urea and 6 M GuHCl was used to study the folding process with the implicit assumption that the denatured protein is completely structureless. However, the assumption has not yet been definitively proven. It has been suggested that some proteins retain considerable residual structure even in the presence of denaturants at high concentrations [11,12]. The presence of any residual ordered structure in a supposedly fully denatured protein would be important because the residual structure could initiate the refolding of the rest of the molecule by maintaining certain hydrophobic or other interactions, which would greatly facilitate the refolding process [13]. Many refolding studies have been made with disulfide bond containing proteins, and in such cases it is even more uncertain whether these proteins still contain ordered structures in strong denaturants. Contradictory results have been presented for RNase A. In an early study, Tanford et al. [14] suggested by intrinsic viscosity measurements that RNase A in 6 M GuHCl is exactly as predicted for a randomly coiled polymer chain, but Hu and Tsou reported that the RNase A denatured in 6 M

GuHCl with their native disulfide bonds is not completely structureless as detected by CD, UV and Fourier transform infrared spectroscopy [15]. In 1992, Sosnick and Trewhella [16] reported a very small  $R_g$  value for reduced-denatured RNase A. They concluded that reduced-denatured RNase A is more compact than a random coil and has a significant amount of regular secondary structure, as deduced from small-angle X-ray scattering measurements and Fourier transform infrared spectroscopy. Soon afterwards Noppert and his co-workers [17] reported that reduced-denatured RNase A is not in a compact state but that reduction of the four disulfide bonds by DTT at 20°C leads to total unfolding and a subsequent temperature increase has no further effect on its dimension. The controversy in the literature may best be resolved using synchrotron small-angle scattering to measure the overall dimensions of a protein molecule in solution. In the present study, the compactness of disulfide intact RNase A and reduced RNase A were investigated using synchrotron small-angle X-ray scattering. The result show that reduced RNase A is more expanded than the non-reduced form but it is in neither random coil nor a compact denatured state.

## 2. Materials and methods

### 2.1. Materials

Bovine pancreatic RNase A (type III-A) was purchased from Sigma and used without further purification. Ultrapure urea was obtained from Nacalai Tesque, Inc., Kyoto, Japan. All chemicals were of analytical grade and were used without further purification.

### 2.2. Reduction of RNase A

Reduced RNase A was prepared according to the procedure described by Kumar et al. [18]. The RNase A was dissolved in 0.1 M Tris-HCl buffer, pH 8.6, containing 8 M urea and 1 mM EDTA, to a final protein concentration of about 10 mg/ml. After the addition of dithiothreitol to a final concentration of 0.15 M, the solution was flushed with nitrogen gas for 3 min and reduction was carried out for 4 h at 25°C. Then, the pH of the mixture was adjusted to 3.0 by the addition of HCl. The reduced RNase A was then dialyzed thoroughly against 0.1 M acetic acid. The dialyzed solution was lyophilized, and the dried protein was stored in a sealed vial filled with nitrogen. The reduced RNase A contains 7.2 reactive SH groups measured by DTNB. The protein concentrations were determined spectrophotometrically using a molar extinction coefficient of 9200 M<sup>-1</sup> cm<sup>-1</sup> at 275 nm for both native and reduced RNase A.

### 2.3. Synchrotron small-angle X-ray scattering measurements

Solution scattering measurements were performed at the beam line 15 small-angle installation of the Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Japan, where a stable beam of photons with a wavelength of 1.5 Å was provided by a bent-crystal horizontally focusing monochromator and a vertically focusing mirror [19]. Samples were irradiated in a quartz cell with 1 mm path length for 300 s for each sample. The temperature of the cell holder was kept at 25°C with circulating water. The background data for the buffer solution and the different urea concentrations were collected before or

\*Corresponding author. Fax: (86) (10) 64872026.  
E-mail: zhoujm@sun5.ibp.ac.cn

after data collection for the protein solution. The small angle X-ray scattering data were corrected for the difference in contrast between the protein and solvent molecules as well as for X-ray absorption by the urea according to standard data provided by Semisotnov (unpublished data). The  $R_g$  values were analyzed by Guinier plot and the compactness was judged by Kratky plot [20].

### 3. Results

#### 3.1. The $R_g$ values of native, denatured and reduced RNase A

The solution X-ray scattering of native RNase A at different concentrations was measured in 50 mM Tris-HCl buffer at pH 8.1 and 25°C. The apparent  $R_g$  values were calculated from a series of Guinier plots at different enzyme concentrations. The  $R_g$  of RNase A was obtained by extrapolating linearly the apparent  $R_g$  values to zero concentration of the enzyme. Curve 1 of Fig. 1 shows the concentration dependence of the  $R_g$  values for RNase A. It is clear that there is no obvious change of  $R_g$  value as the enzyme concentration was increased and that the  $R_g$  value at zero concentration is  $15.4 \pm 0.13$  Å. The concentration dependence of the  $R_g$  values for RNase A denatured in 6 M urea was also determined as shown in curve 2 of the same figure. The  $R_g$  of 6 M urea-denatured RNase A is also almost independent of the protein concentration with a value of  $15.5 \pm 0.11$  Å at zero concentration. Fig. 1, curve 3 shows the concentration dependence of the  $R_g$  value of reduced RNase A in 50 mM Tris-HCl buffer pH 8.1 containing 20 mM DTT and 6 M urea. The  $R_g$  increases slightly with increasing enzyme concentration and the  $R_g$  value at zero concentration is  $24.0 \pm 0.1$  Å. This value is in good agreement with the results reported by Sosnick and Trewhella [16].

#### 3.2. Changes in compactness of RNase A during denaturation by urea

The  $R_g$  values of RNase A denatured in different concentrations of urea were measured. The dependence of  $R_g$  on urea concentration is shown in Fig. 2. There is no obvious change of the  $R_g$  for RNase A with intact disulfide bonds (Fig. 2, curve 1) during urea denaturation. The Kratky plots show that both native and 8 M urea-denatured enzyme are in

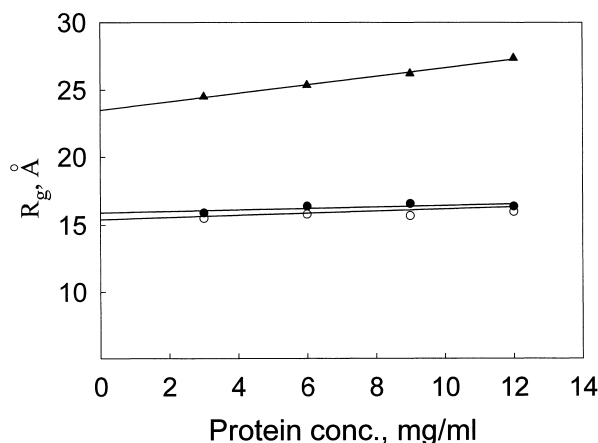


Fig. 1. Concentration dependence of the apparent  $R_g$  values obtained from the Guinier plot at different protein concentrations. Lower curve, RNase A in 50 mM Tris-HCl buffer pH 8.1; middle curve, 6 M urea-denatured RNase A in 50 mM Tris-HCl buffer pH 8.1; top curve, 6 M urea-denatured reduced RNase A in 50 mM Tris-HCl buffer pH 8.1 containing 20 mM DTT.

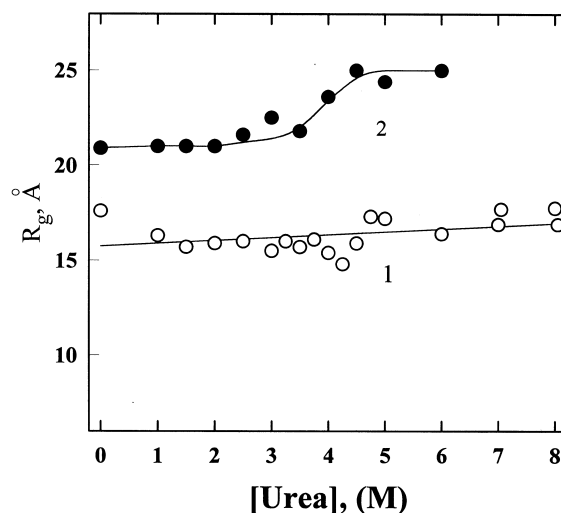


Fig. 2. Urea-induced equilibrium transitions of the  $R_g$  value. Curve 1, disulfide intact RNase A; curve 2, reduced RNase A. The buffer conditions were the same as in Fig. 1.

a globular state (Fig. 3, curves 1 and 2). However, the  $R_g$  of reduced RNase A is larger than that of disulfide intact RNase A in the absence of urea and increases gradually at urea concentrations higher than 2.5 M, reaching 24 Å in 6 M urea (Fig. 2, curve 2). The Kratky plots show that reduced RNase A is a partially coiled structure even in the absence of urea and is a random coil in the presence of 6 M urea (Fig. 3, curves 3 and 4).

Contradictory results for the  $R_g$  of reduced RNase A have been reported in the literature: Sosnick and Trewhella [16] found that the protein has the same dimensions as the native protein ( $R_g = 15$  Å) in the presence of a strong reducing agent, 100 mM DTT at 45°C, and expands further with increasing temperature. However, Noppert and his co-workers [17] re-

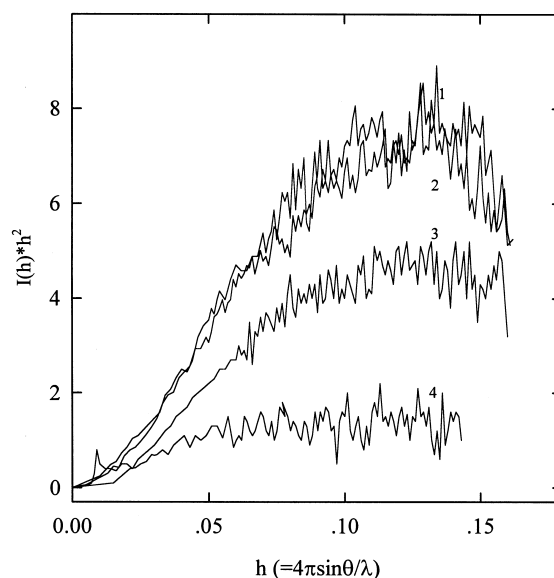


Fig. 3. Kratky plots for RNase A. 1, Disulfide intact RNase A; 2, disulfide intact RNase A denatured in 8 M urea; 3, reduced RNase A; 4, reduced RNase A denatured in 6 M Urea. The buffer conditions were the same as in Fig. 1.

ported that reduction of the four disulfide bonds by DTT at 20°C leads to total unfolding and that increasing the temperature has no further effect on its dimension. They suggested that reduced-denatured RNase A has a random coil-like conformation.

#### 4. Discussion

##### 4.1. The compact globularity of RNase A molecules in urea-denatured state is maintained by its disulfide bonds

Denaturation of disulfide intact RNase A in urea and guanidine hydrochloride has been extensively studied. The enzyme is fairly resistant to denaturation by low concentrations of denaturants. The unfolding becomes significant beginning at a guanidine concentration of 3 M and approaches completion at 6 M. The unfolding of disulfide intact enzyme is a biphasic kinetic reaction as followed by intrinsic fluorescence at 305 nm and ultraviolet absorbance at 287 nm [21]. The disappearance of different secondary structures requires different concentrations of denaturants as reported by Chen and her co-workers using Fourier transformed infrared spectra [22]. Although there is some ordered structure remaining in the RNase A molecule with native disulfide bonds [15], the secondary and tertiary structures of the enzyme are seriously damaged at high denaturant concentrations as monitored by fluorescence, absorbance and infrared spectra. The midpoint of unfolding RNase A at pH 8.1 is about 7 M urea [23]. However, both native and 8 M urea-denatured RNase A are in compact globular shape as indicated by their Kratky plots (Fig. 3, curves 1 and 2) with apparent  $R_g$  values of 15–17 Å, indicating that the disulfide bonds are able to keep the denatured enzyme in an apparent globular state. This is a denatured globular state with secondary and tertiary structure damaged. Therefore, it is different from the ‘compact denatured states’ of protein reported by Lattman et al. [24]. They proposed a model for the conformation of compact denatured states of globular proteins which corresponds to the ‘molten globule state’ having the backbone chain with the conformation of the native protein and the disorder in the compact denatured state only in the side chains. We suggest that the disturbed apparent compact structure of disulfide intact enzyme in the presence of high concentrations of denaturants is not its folding intermediate but a residual structure that could be the origin of hydrophobic collapse, which drives the initiation of protein folding.

##### 4.2. Reduced RNase A is in a more expanded state than non-reduced enzyme and this state is neither a random coil nor a compact denatured state

The  $R_g$  of reduced RNase A in 50 mM Tris-HCl buffer pH 8.1 containing 20 mM DTT is more expanded than the molecule with native disulfide bond as characterized by the Kratky plots (Fig. 3, curve 3). The reduced enzyme is further expanded in the presence of higher concentrations of urea until the  $R_g$  reaches  $24 \pm 0.10$  Å in 6 M urea. These results are consistent with the  $R_g$  for non-reduced (15 Å) and reduced enzyme ( $R_g = 24$  Å) in 6 M guanidine reported by Sosnick and Trehwella, but our data are not in agreement with their result that the  $R_g$  is also 15 Å at 45°C for the reduced enzyme in the absence of denaturants. As pointed out by Nöppert et al. [17], Sosnick et al. probably underestimated the  $R_g$  value of the reduced enzyme because reduction of the enzyme was not

complete in their experimental conditions. Since the four disulfide bridges of this enzyme can only be reduced when the protein is unfolded, the protein must be kept in a high concentration of denaturant (8 M urea or 6 M GuHCl) for a long time in the presence of thiol reagents such as DTT. The reduced RNase A used in the experiments could be refolded by removing the denaturants from the reducing solution and maintaining the –SH group in the reduced state by the addition of an appreciable amount of DTT. Our experiment effectively prevents re-oxidation of the reduced –SH groups by the presence of 20 mM DTT during the whole experimental process. We suggest that the underestimation of the  $R_g$  for reduced RNase A is due to incomplete reduction of the disulfide bonds and that the overestimation of the  $R_g$  of reduced RNase A is probably due to incomplete refolding of the denatured chains after reducing all of the disulfide bonds.

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